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Please forward:
1. Proceedings of the 45th ASMS conference onMass spectrometry and allied Topics, Palm Springs, June 1-5, 1997, p. 907, Siegel et al
2. Proceedings of the 44th ASMS conference on mass spectrometery and allied topics, Portland, Or. May 12-16, 1996, p. 1424, Siegel et al.
3. Protein Science, 3, 81, (1994), Hutchens et al
4. Rapid Commun. Mass Spectrom. 7, 576 (1993).

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. 7, 576–580 (1993)

New Desorption Strategies for the Mass-Spectrometric Analysis of Macromolecules†

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We present two new desorption strategies for the mass spectrometric analysis of macromolecules. These desorption strategies are based on the molecular design and construction of two general classes of sample 'probe' surfaces. The first class of surfaces is designed to enhance the desorption of intact macromolecules presented alone (neat) to the surface; we call this surface-enhanced neat desorption (SEND). The availability of probe surfaces derivatized with, or composed of, multiple types and defined numbers of energy-absorbing molecules will facilitate investigations of energy transfer and desorption/ionization mechanisms. The second class of probe surfaces is designed to enhance the desorption of specific macromolecules captured directly from unfractionated biological fluids and extracts; we call this surface-enhanced affinity capture (SEAC). Use of these new probe surfaces as *chemically defined* solid-phase reaction centers will facilitate protein discovery through molecular recognition *in situ*, and also macromolecular structure analysis through the sequential chemical and/or enzymatic modification of the adsorbed analyte *in situ*. Specific examples of laser-assisted SEND and SEAC time-of-flight mass spectrometry are presented to illustrate the potential for increased selectivity, analyte detection sensitivity, and mass measurement accuracy.

The introduction of matrix molecules to assist the laser-induced desorption/ionization (MALDI) of intact macromolecules has enabled relatively small quantities of large nonvolatile biopolymers to be evaluated by time-of-flight (TOF) mass spectrometry.[1] Many new types of investigations have been enabled by this technology.[2]

The ability to measure precise *differences* in molecular mass enables problems associated with unknown protein structures to be solved more readily.[3,4] Thus, the future of biological mass spectrometry will be defined by simpler and more sensitive means to monitor specific changes in protein and peptide mass.

Our laboratory has developed MALDI-TOF to explore various specific ligand–ligate interactions and to analyze protein structure;[3–10] both approaches have been based largely on biochemical reactions performed directly on the probe surface (i.e., *in situ*) to exploit the availability of analyte not consumed by previous laser-induced desorption events. However, the required presence of a large molar excess of acidic matrix and the random deposition of *embedded* macromolecular analytes in and around an undefined array of matrix crystals has limited the use of the probe surface as an effective stationary phase from which to conduct, by differential mass spectrometry, a sequential *in situ* approach to the investigation of protein structure and function.

An effective mass spectrometric strategy for protein structure analysis may be realized if each of several diagnostic alterations in mass can be brought about as a result of controlled chemical and/or enzymatic modification events performed directly and sequentially *in situ*, i.e., directly on the probe surface. In fact, alterations in both covalent and noncovalent structures (including higher order macromolecular assemblies) may be evaluated more readily once (i) conditions are defined to minimize or eliminate the negative influence of matrix molecules, and/or (ii) an active, rather than passive, means of protein deposition on the probe surface is developed. Our previous experience with the matrix-assisted laser desorption of peptides and proteins directly from electrophoretic gels and various membrane protein blots has provided less than desirable results (e.g., low signal yield, low resolution). Thus, we have attempted to advance protein structure and function analyses based on laser desorption TOF-MS by redesigning probe surfaces (or by depositing specifically constructed macromolecular structures on the surface of the desorption probe element) to contain the necessary density of energy-absorbing molecules so that the need for matrix can be eliminated.

We introduce here methods for desorption of large molecular weight biopolymers into the gas phase (without fragmentation) using surfaces chemically defined to contain predetermined numbers of bound energy-absorbing molecules. We call the general approach surface-enhanced neat desorption (SEND) time-of-flight mass spectrometry.

To enhance our investigation of bioregulatory molecular recognition events and to eliminate the suppression of molecular desorption/ionization often observed with complex mixtures, we have also designed a class of probe surfaces to be used as affinity capture devices to augment the detection of trace macromolecules present in unfractionated biological samples, particularly *in vivo*. To distinguish this approach from other indirect strategies we suggest the term laser-assisted surface-enhanced affinity capture (SEAC) time-of-flight mass spectrometry.

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EXPERIMENTAL

Laser desorption time-of-flight mass spectrometry

Laser desorption (both MALDI and SEND) time-of-flight mass spectra were collected on a modified Vestec Model VT2000 (Houston, TX, USA) exactly as described previously.[6] Spectra shown were collected in the positive-ion mode. Real-time signal averages of 50 laser shots (to a single spot, approx. 100 × 200 μm) were used to generate each spectrum.

Composition of mass spectrometer probe platform elements and/or various sample-presenting surfaces

Sample-presenting surfaces are defined as the surface of the solid phase used to actively dock or to passively deposit molecular analytes in a manner required for successful desorption during subsequent presentation to laser irradiation *in vacuo*. Such enabling sample-presenting surfaces may be comprised of the probe platform element itself (after chemical modification of the surface) or other structures placed on the surface of the otherwise inert probe platform (foundation) alone. That is, the probe element surface itself may or may not be transformed into an active sample-presenting surface. The compositions of the entire probe element, its surface, and/or additional structures with surfaces used to absorb energy and/or present analytes for desorption were varied extensively, of which variations only specific examples are presented here. Polypropylene or polystyrene were melted in an open flame and deposited as a thin layer on a 2 mm diameter stainless-steel probe element and allowed to cool. Solid glass rod or solid nylon filaments (up to 1.5 mm diameter) were cut into 1 cm segments and inserted into the stainless-steel probe support. Porous agarose (6%) was crosslinked with divinyl sulfone (Fluka Chemical, Ronkonkoma, NY, USA) as described previously.[10]

Synthetic peptide analyte mixtures

Synthetic peptides including the human histidine-rich glycoprotein peptide $(GHHPH)_5G$ (2903.3 Da), human estrogen receptor dimerization domain D473-L525 (ERDD; 6168.4 Da), and human progesterone receptor C-terminal fragment (PR-CTF; 8228.9 Da) were synthesized, purified, and characterized as described previously.[10]

Evaluation of probe platform elements and/or various sample-presenting surfaces by MALDI

Combinations of synthetic peptides (2–5 pmol each) were mixed with 1 μL of sinapinic acid (in 30% methanol with 0.1% trifluoroacetic acid) on each of the sample-presenting surfaces evaluated. In this case, the sinapinic acid was added and used as defined previously[2] by the MALDI process to embed the analyte(s) for protection (i.e., desorption/ionization in the intact state).

Surface-enhanced neat desorption (SEND)

The following two model cases are presented as representative examples of converting inert surfaces to modified surfaces that function to enhance neat desorption of intact macromolecules. It should be emphasized that in the case of SEND, the sample (analyte) is added alone (neat) to the chemically defined desorption surface; no other matrix is added.

In the first case, α-cyano-4-hydroxycinnamic acid[11] (Aldrich Chemical, Milwaukee, WI, USA) was dissolved in methanol and mixed with crosslinked agarose beads (75–300 μm diameter) activated with *N*-hydroxysuccinimide at 23 °C for 2 h. The unbound energy-absorbing molecules were removed by washing (5 washes each with at least 10 gel volumes) with methanol until the wash solution contained no UV/VIS absorbance (i.e., all unbound energy-absorbing material was removed). A 1 μL slurry (50:50, v/v) of the modified agarose surface structures (<15 nmol of energy-absorbing molecules per μL gel), to function as the energy-absorbing/sample-presenting surface, was placed on the inert probe element surface with 2 pmol of horse-heart myoglobin; laser irradiation of a single bead of modified agarose was sufficient to obtain a signal.

The second specific example of converting an inert surface for the neat desorption of protein was chosen because it represents the use of a class of energy-absorbing molecules that we found *not* to be an effective 'matrix' when presented in excess as free molecules in solution together with the analyte protein (i.e., as in MALDI). Cinnamamide (Aldrich) was dissolved in isopropanol + 0.5 M sodium carbonate (3:1, v/v) and mixed with divinylsulfone (Fluka) activated Sepharose 6B (45–145 μm diameter) (Pharmacia Biotech, Piscataway, NJ, USA) at 23 °C for 2 h. The excess (i.e., unreacted) cinnamamide was washed away with isopropanol until the wash solution had no UV absorbance characteristic of cinnamamide. One μL of an aqueous slurry (50:50, v/v) of the modified surface structures were deposited on the inert probe element surface. One μL of peptide mixture (containing estrogen receptor dimerization domain and progesterone receptor C-terminal fragment) was added to the sample-presenting surface structure. No free matrix, of any kind, was added to the probe surface.

Surface-enhanced affinity capture (SEAC)

The following example was chosen to illustrate the use of an affinity-capture device on a sample-presenting surface to retrieve and present one or more desired analyte molecules. Analyte capture may be accomplished in either of two different modes: either directly *in situ* (i.e., when the probe surface is composed of and defined to be the affinity-capture sample-presenting surface), or indirectly (i.e., off-site and prior to presentation to the inert surface of the probe element). In this particular case, after placing the affinity-capture device onto the inert probe platform (glass), exogenous matrix was added as described typically for MALDI. Preterm infant urine (1 mL) containing a known quantity (0.6 nmol) of human glycoprotein lactoferrin (MW 80 kDa) was mixed, at 23 °C for 15 min, with affinity-capture devices comprised of agarose beads (approx. 40–120 μm diameter) with sample-presenting surfaces defined by covalently immobilized single-stranded DNA (0.5–1.0 mg DNA per mL gel) (GIBCO BRL, Gaithersburg, MD, USA). The beads were washed successively with 2 volumes (500 μL each) of the 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-

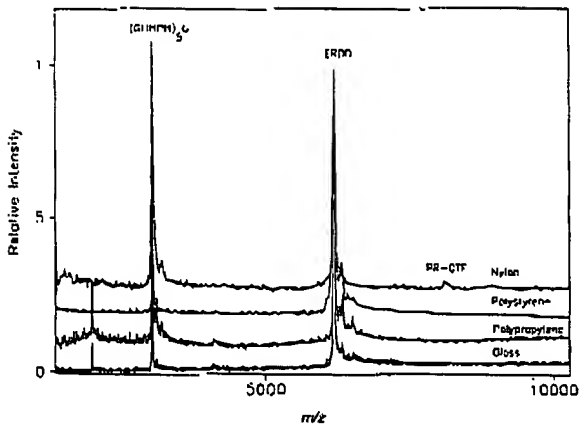

Figure 1. A composite of MALDI-TOF mass spectra of peptide mixtures (see Experimental) using sinapinic acid as the matrix. Probe surfaces included glass, polypropylene-coated stainless steel, polystyrene-coated stainless steel, and pure nylon. Labeled molecular ions are all $[M+H]^+$ species.

ethanesulphonic acid (HEPES) buffer, pH 7.4, then with HEPES buffer containing 3 M urea (pH 7.4), and finally with water. One μL of an aqueous slurry (50:50 v/v) was mixed with 1 μL sinapinic acid (30% methanol, 0.1% trifluoroacetic acid) on a glass probe element. Laser irradiation of a single SEAC device was found to be sufficient to obtain good quality spectra (av. 50 shots).

RESULTS

Figure 1 shows that macromolecular analytes (proteins or peptides) could be desorbed efficiently from probe elements composed entirely of insulating materials like nylon, polystyrene, polypropylene, and glass by the normal MALDI process. No apparent fragmentation or evidence of surface-charging phenomena was observed. Some differences in desorption selectivity were observed among the various surfaces tested (for example, see the 8228 Da PR-CTF peak).

Figure 2 shows one example of SEND of myoglobin from a surface structure containing bound α-cyano-4-hydroxycinnamic acid as the energy-absorbing molecules. The peak appeared symmetrical with no evidence of matrix adducts (mass resolving power ranged from 170 to 480), fragmentation, or low molecular weight ions other than those observed from buffer contamination. Note the lack of multiply charged molecular ions beyond $[M+2H]^{2+}$ (a characteristic feature of the use of α-cyano-4-hydroxycinnamic acid as a matrix in MALDI).[11] The lack of observed analyte–matrix adducts, the reduced multiple-charging effect, and the absence of free matrix ions—features typically observed by the MALDI process—distinguish the SEND process from the MALDI process.

Figure 3 shows an even more striking example of SEND. In this case, the energy-absorbing molecule chosen, cinnamamide, was not effective as a matrix when used in conventional MALDI. The estrogen receptor dimerization domain (6168.4 Da) and the progesterone receptor C-terminal fragment (8228.9 Da) were used as test analytes. The lower profile of Fig. 3 is shown, to demonstrate that peptide signals were *not* observed by MALDI when the free cinnamamide matrix and analyte were mixed in solution. However, when cinnamamide was covalently bonded to the sample-presenting surface, the resulting structure was transformed to enhance desorption of the peptides in a neat form with no evidence of fragmentation or adduct formation. The mass resolution ($M/\Delta M = 380$) was sufficient to resolve matrix adducts (M + 147.2 Da) if they had been formed.

Figure 4 shows one example of surface-enhanced affinity-capture (SEAC) laser desorption time-of-flight mass spectrometry to augment the detection of an 80-kDa glycoprotein not observed by MALDI-TOF-MS. Fig. 4(a) shows the mass spectrum of an infant urine sample obtained by MALDI. In addition to the absence of good quality molecular ion signals at any mass, we were not able to detect, with any certainty, the relatively low quantity (ca 1.5–3 nmol/mL) of intact lactoferrin known to be present[12,13] in this infant urine. The spectrum shown in Fig. 4(a) is typical (with the number of experiments being ca 48) of that obtained for infant urine. It is common to find evidence of global signal (molecular ion) suppression, particularly at higher mass

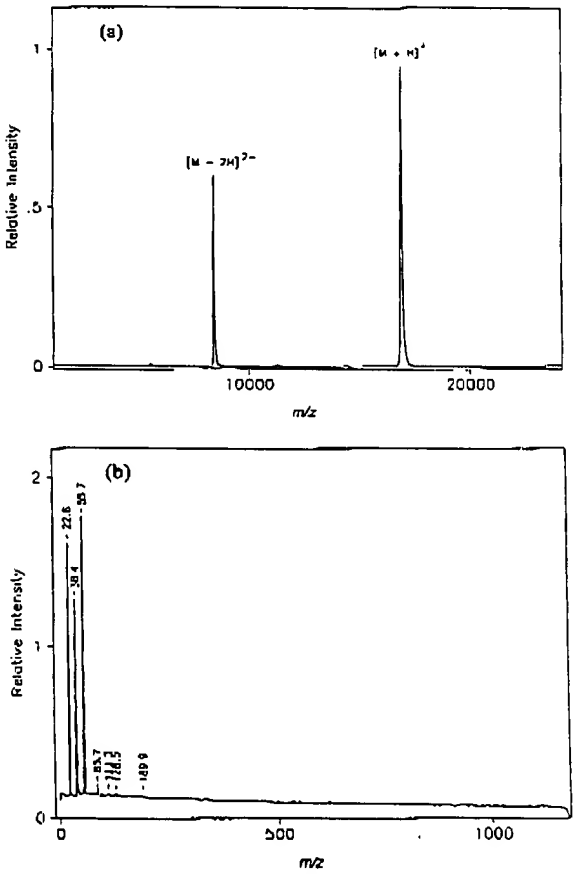

Figure 2. (a) An example of SEND-TOF mass spectrometry showing horse-heart myoglobin desorbed from a sample-presenting surface (2 pmol myoglobin added) containing *bound* α-cyano-4-hydroxycinnamic acid to enhance desorption. The myoglobin sample was added alone (without matrix) to the modified sample-presenting surface. Only the $[M+H]^+$ and the $[M+2H]^{2+}$ molecular ions were observed. (b) The low mass region of this spectrum (note the absence of matrix ions).

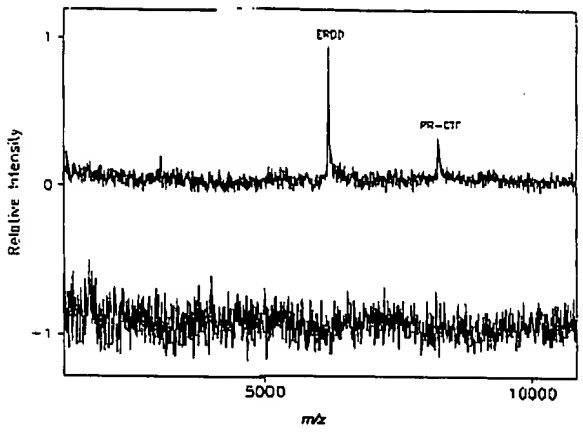

Figure 3. An example of SEND-TOF mass spectrometry showing a peptide mixture (ERDD and PR-CTF) desorbed from a sample-presenting surface enhanced with bound energy-absorbing molecules, in this case cinnamamide (top profile); only the $[M+H]^+$ molecular ions were observed. For comparison, the peptide mixture (ERDD and PR-CTF) was also deposited on a stainless-steel probe element platform in the presence of free cinnamamide added as a saturated solution in 30% methanol with 0.1% TFA (MALDI; lower profile). Note the absence of molecular ion signals with cinnamamide used as a matrix for MALDI.

values, in the case where the biological sample contains either high quantities of large molecular mass molecules or a large number of molecules representing a continuum of mass (e.g., preterm infant urine). In contrast, when a sample of infant urine containing a 5-fold lower concentration of lactoferrin (0.6 nmol/mL) (Fig. 4(b)) was evaluated by the SEAC process, the resulting TOF mass spectrum was relatively clean and unambiguous with a significant signal of lactoferrin observed. The affinity capture of analyte, removal of contaminants, and transfer to the probe surface was specific and efficient. The quality of signal was apparently not compromised by the presentation of analyte on a sample-presenting surface that was not flush with the probe element surface.

Other examples of affinity capture and neat desorption (including combined SEAC and SEND) are in preparation.

DISCUSSION

A matrix, as used in MALDI, is defined as a substance from *within* which something else (i.e., analyte) originates or develops, i.e., a material in which the analyte is *enclosed* or *embedded* for protection. In contrast, SEND has been demonstrated to be, and is hereby defined as, a *surface-dependent* event (SEAC may be used with or without matrix). Furthermore, and more importantly, SEND and SEAC enable laser-assisted macromolecular desorption processes that are dependent on and varied by the design and architecture of chemically and/or biologically defined surface structures.

The benefits associated with SEND can be observed in the spectrum of myoglobin. The absence of a matrix adduct and the absence of small molecular mass fragments suggests a desorption mechanism for SEND unlike the one proposed for MALDI; additional experiments are needed to verify the desorption/ionization process involved.

The benefits associated with SEAC were apparent in the extraction of lactoferrin from urine. We have observed other examples where SEAC allows the successful presentation and detection of attomoles of analyte (data not included here).

In the case of protein structure analysis, beyond evaluation of primary structure (sequence) and post-translational modifications, however, are questions concerning secondary, tertiary, and quaternary structures. The availability of mass spectrometric probes with surfaces enhanced for neat desorption (SEND) and affinity capture (SEAC) should enable a variety of new opportunities to investigate protein structure and function.

We have used probe element surfaces and sample-presenting surfaces varying widely in composition (including glass, ceramics, teflon-coated magnetic materials, organic polymers, and native biopolymers), in diameter, geometry, surface area, and density of immobilized energy-absorbing molecules and/or affinity-

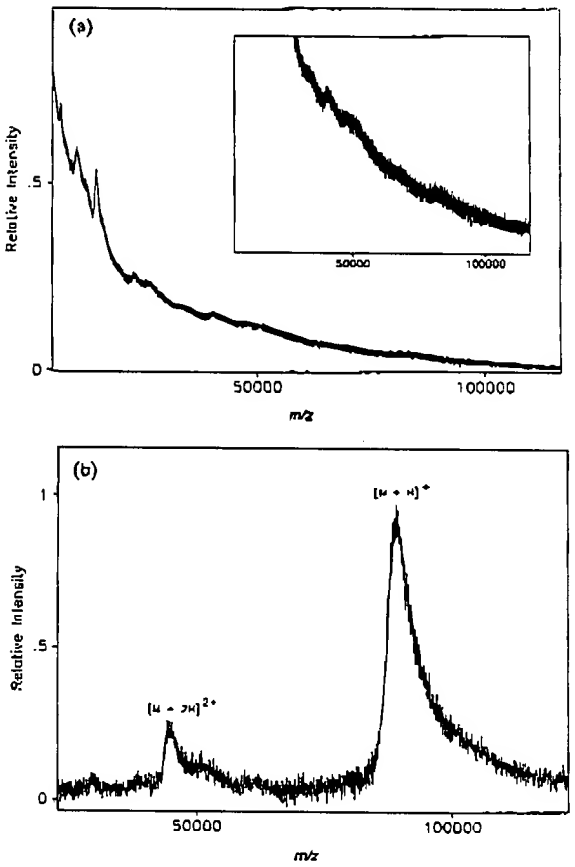

Figure 4. An example of SEAC laser desorption TOF mass spectrometry. (a) The MALDI-TOF mass spectrum obtained with urine (1.5–3 nmol/mL) applied directly to the unactivated probe surface with sinapinic acid as the matrix. (b) TOF mass spectrum showing human lactoferrin captured from a preterm infant urine with a 5-fold lower concentration of lactoferrin. The sample-presenting surface for this SEAC experiment was composed of porous agarose with immobilized single-stranded DNA as the affinity-capture device; the inert probe element was glass. In this case, sinapinic acid was added as matrix.

capture devices. Some of the surface-exposed affinity-capture reagents used thus far include proteins (e.g., immunoglobulins), intact protein subunits, specific protein surface domains, nucleic acids (e.g., DNA), various transition metal ions, carbohydrates, lectins, dyes, reducing reagents, and various lower-affinity binding reagents involving cationic, anionic, and hydrophobic interactions. Specific examples of each are to be presented in detail elsewhere.

Protein structure analysis by the method of *differential* mass spectrometry has already been demonstrated as a valid experimental approach. This approach remains one of the only viable options when quantities of protein are severely limited (<pmol). The new probe surfaces presented here should be considered and exploited as solid-phase reaction sites. These surfaces should improve the sensitivity and accuracy of the protein discovery process by enabling low affinity molecular recognition events to be performed directly *in situ*. Extensive macromolecular structure analysis through the sequential chemical and/or enzymatic modification of the adsorbed analyte *in situ* is also possible now that the interference from the matrix can be eliminated. The availability of probe surfaces derivatized with, or composed of, multiple types and defined numbers of energy-absorbing molecules should also facilitate the use of alternative methods of energy transfer and less ambiguous investigations of desorption/ionization mechanisms.

CONCLUSION

The appropriate design and construction of chemically defined probe surfaces can enable the selective capture of specific analytes (SEAC) and enable the neat desorption of intact macromolecules (SEND) for analysis by time-of-flight mass spectrometry. Differences in sample preparation (effective concentrations and compositions, application to the probe surface, physical state of the adsorbed analyte molecules, location from the probe surface at the point of desorption) and quality of mass spectra distinguish SEND from MALDI. Certain of these differences (e.g., the absence of detectable ions from the energy-absorbing surface structures and absence of matrix-adduct formation) suggest that different desorption/ionization mechanisms may be involved for these two different processes.

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